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Citation for published version:
Rocha, AS, Vidal, VPI, Mertz, M, Kendall, T, Charlet, A, Okamoto, H & Schedl, A 2015, 'The angiocrine factor Rspondin3 is a key determinant of liver zonation', *Cell Reports*, vol. 13, no. 9, pp. 1757–1764. https://doi.org/10.1016/j.celrep.2015.10.049

Digital Object Identifier (DOI):
10.1016/j.celrep.2015.10.049

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Cell Reports

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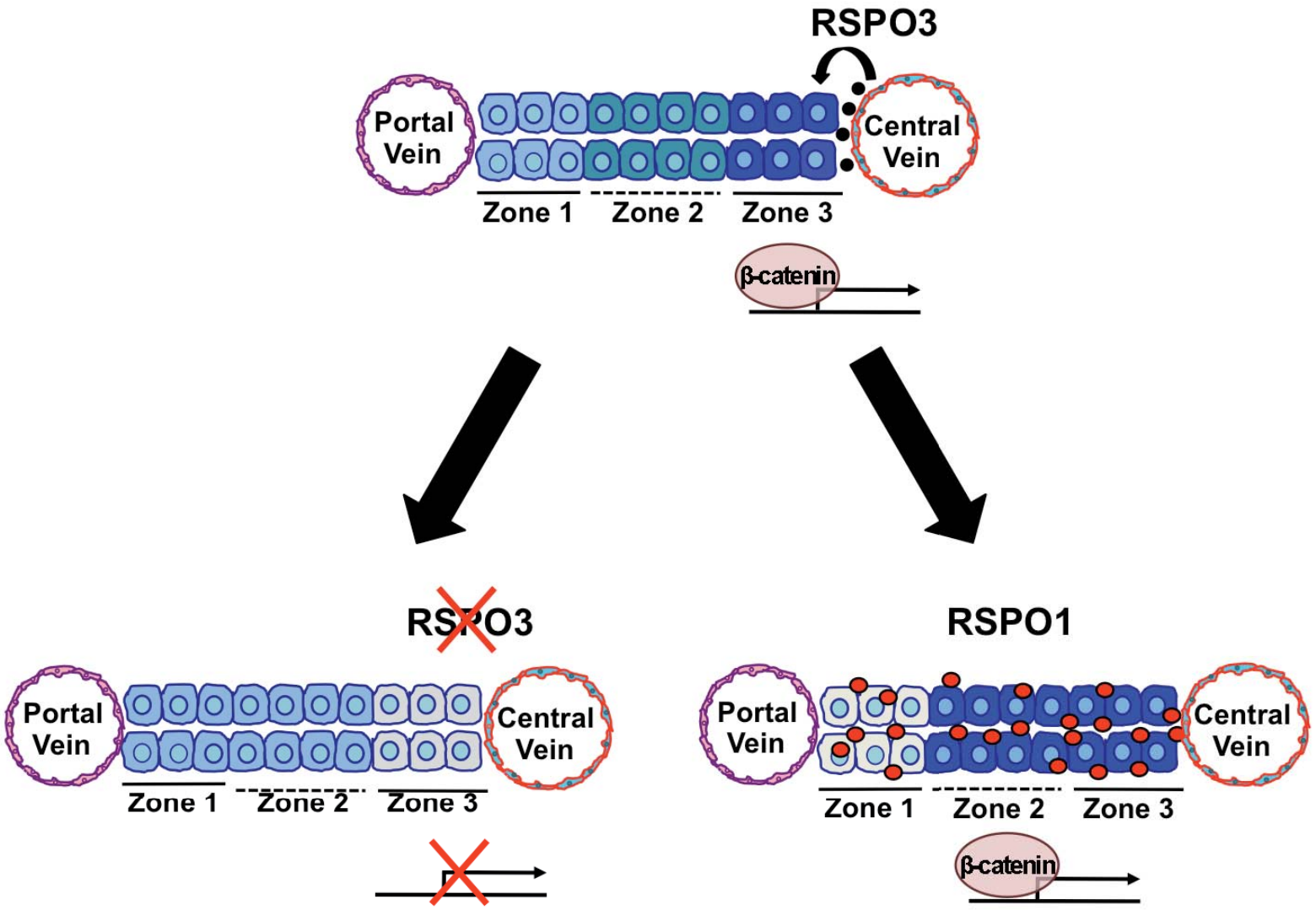
Download date: 05. May. 2023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Zone 3
β-catenin

The angiocrine factor Rspondin3 is a key determinant of liver zonation

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Key words: Liver zonation, homeostasis, Rspondin, β-catenin signaling

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Abstract

Liver zonation, the spatial separation of different metabolic pathways along the liver sinusoids, is fundamental for proper functioning of this organ and its disruption can lead to the development of metabolic disorders such as hyperammonemia. Metabolic zonation involves the induction of β-catenin signaling around the central veins, but how this patterned activity is established and maintained is unclear. Here we show that the signaling molecule *Rspondin3* is specifically expressed within the endothelial compartment of the central vein. Conditional deletion of *Rspo3* in mice disrupted activation of central fate demonstrating its crucial role in determining and maintaining β-catenin dependent zonation. Moreover, ectopic expression of *Rspo1*, a close family member of *Rspo3*, induced the expression of pericentral markers demonstrating Rspondins to be sufficient to imprint a more central fate. Thus *Rspo3* is a key angiocrine factor that controls metabolic zonation of liver hepatocytes.

Introduction

The liver is a central organ responsible for the metabolism of dietary compounds, regulation of blood glucose levels, bile synthesis and biotransformation of xenobiotics and endogenous by-products of metabolism. During development, the fetus is able to rely on the metabolic activity of the maternal liver. In the neonatal period however, the liver needs to undergo several changes until reaching full maturation at weaning (Grijalva and Vakili, 2013). This includes specialization of groups of hepatocytes, a process involving the activation of specific enzymes that allow effective execution of metabolic tasks.

The functional unit of the liver consists of the hepatic lobule, which includes a central vein connected to four to six portal triads via the hepatic plates. Hepatocytes located within these functional units perform specific functions depending on their location within the lobule. Three zones of hepatocytes can be distinguished: hepatocytes located closest to the periportal triad (zone 1) are exposed to blood with the highest oxygen and nutrient concentration and perform the majority of the liver's metabolic functions including glycogen synthesis, gluconeogenesis, protein synthesis and lipid metabolism. The hepatocytes closest to the central veins are located in zone 3 and are specialized for biotransformation reactions, glycolysis and urea synthesis. Hepatocytes in zone 2 display an intermediate phenotype executing both periportal and pericentral functions (Torre et al., 2010).

On the molecular level, liver zonation is governed by canonical Wnt/β-catenin signaling. Its activity is highest around the central vein and nuclear β-catenin directly regulates the activation of genes involved in glutamine synthesis and drug metabolism (Benhamouche et al., 2006; Burke et al., 2009; Sekine et al., 2006). Consistent with this role in regulating the central fate, activation or attenuation of β-catenin signaling causes expansion or diminution of central markers, respectively. Although the role of Wnt/β-catenin signaling is well established, the factors determining when and where this pathway is active are still elusive.

The Rspondin family of Wnt agonists has recently been demonstrated to play a major role in determining the levels of Wnt/β-catenin activity (Yoon and Lee, 2012). Upon binding to its receptors – the LGRs – Rspondins prevent membrane clearance of Wnt receptors thereby promoting Wnt signaling activity (Clevers et al., 2014). This signaling module has recently attracted a lot of attention due to its role in stem cell biology, with a particular emphasis on LGR5 that has been shown to label stem cells in various tissues, both under physiological and pathological conditions (Clevers et al., 2014). The liver is no exception and Huch *et al* recently demonstrated that LGR5 labels a damage-activated stem cell population (Huch et al., 2013). Interestingly, LGR5 was shown to be differentially expressed between periportal and pericentral hepatocytes (Braeuning et al., 2006). LGR5 mRNA was exclusively identified

in pericentral hepatocytes in accordance with the observed Wnt/β-catenin signaling activity in adult livers. This observation led us to investigate whether Rspondins play a role in determining Wnt/β-catenin signaling activity, and therefore in regulating liver metabolic zonation.

Results

Rspo3 is an endothelial specific marker demonstrating lobular zonation.

Rspondin expression has previously been analyzed during development, but no liver-specific expression has been reported (Nam et al., 2007). Because liver zonation is established soon after birth and maintained through adulthood, we began by characterizing their expression profile at later stages of liver development, namely at E17.5, at P10 as a representative time point for the postnatal period and in adulthood (8 weeks of age). RT-PCR analysis demonstrated *Rspo1* and *Rspo3* to be expressed while *Rspo2* and *Rspo4* were undetectable (Figure S1A). Although *Rspo1* was easily detectable by RT-PCR, we failed to observe a specific expression pattern by *in situ* hybridization (data not shown). The lack of involvement of *Rspo1* was further confirmed in knockout animals that showed overall normal liver histology and maintained the expression of zonation markers, such as Glutamine Synthetase (GS) (Figure S1B and C). Given these observations we focused our attention on *Rspo3*.

In situ hybridization for *Rspo3* demonstrated an exclusively endothelial-specific expression pattern as early as E17.5. Interestingly, the signal seemed to be restricted to the central vein, whereas endothelial cells localized at sites of ductal plate differentiation (identified by high expression of Keratin 18) were consistently negative for *Rspo3* (Figure1A). The central vein specific expression profile of *Rspo3* was maintained in the perinatal period and adulthood. To confirm that *Rspo3* expression was indeed endothelial we FACS sorted CD31+ cells from adult livers and compared the expression profile between sorted cells and whole liver. We observed a dramatic enrichment of *Rspo3*, as well as the *Wnt* ligands *Wnt2* and *Wnt9b* in the endothelial compartment (Figure 1C). Hepatocytes surrounding the *Rspo3* positive veins displayed a strong expression of GS, a *bona fide* Wnt/β-catenin target in hepatocytes (Figure 1B).

Rspo3 is required for the onset and maintenance of metabolic zonation.

Metabolic zonation has been suggested to involve position-specific differentiation along the lobule that begins after birth and becomes fully established soon thereafter (Torre et al.,

2010). The close association between the *Rspo3* expression domain and that of central vein hepatocyte identity suggested that *Rspo3* might be responsible for the functional patterning of pericentral hepatocytes by driving Wnt/ β-catenin signaling. *Rspo3* knockout mice die around E9.5 due to defects in placenta development (Aoki et al., 2007; Kazanskaya et al., 2008), thus precluding analysis at later time points. Therefore, to functionally evaluate an involvement of this gene in liver zonation, we generated *cCAG-CreERT2: Rspo3$^{fl/fl}$* mice that permit an inducible, ubiquitous deletion of *Rspo3* at any given time point. We administered tamoxifen to females at E16.5 days of pregnancy to abolish *Rspo3* expression during late development and perinatal stages (Figure 2A & B). Livers were analyzed at P10, a time point where zonation in wildtype mice is fully established. qPCR analysis confirmed efficient *Rspo3* deletion (98% reduction; p=0,0002). Loss of *Rspo3* had a profound impact on Wnt/ β-catenin signaling, as evidenced by the dramatic reduction of the canonical Wnt/β-catenin targets *Axin2* (90% reduction, p=0,0006) and *LGR5* (98% reduction, p=0,0003) (Figure 2C). The expression of endothelial specific *Wnt2* and *Wnt9b* were not affected by *Rspo3* deletion indicating that the observed phenotype is not due to the absence of the Wnt source, but rather *Rspo3* itself (Figure 2C). Immunohistochemical analysis revealed a complete loss of expression of the pericentral zonation marker GS, demonstrating an absolute requirement for *Rspo3* in establishing pericentral gene expression (Figure 2D). Similarly, expression of *Oat* and *Cyp7a1*, two other genes restricted to pericentral hepatocytes (Braeuning et al., 2006) was dramatically reduced (Figure 2E). Moreover, the periportal genes *Cyp2f2* and *Hsd17* showed a dramatic upregulation, further confirming profound defects in the establishment of liver zonation (Figure 2G). E-cadherin, a marker of adult periportal hepatocytes, was not yet fully zonated in P10 wildtype livers and - as evidenced in Figure 2F - *Rspo3* deletion did not result in dramatic changes in its expression pattern. The molecular changes were not due to apoptosis of pericentral hepatocytes or to altered liver growth (Figure S2A and B).

Central vein specific expression of *Rspo3* is found throughout life. To address whether continuous signaling may be required to maintain liver zonation, we induced *Rspo3* deletion in 8-week old animals (Figure 3A and B). qPCR analysis demonstrated a 50% reduction in *Rspo3* levels following tamoxifen induction indicating only partial deletion of the *floxed* allele. Importantly, a similar downregulation of the Wnt/β-catenin targets *Axin2* and *LGR5* was observed (Figure 3C). *Rspo3* deletion had no impact on *Wnt2* and *Wnt9b* expression (Figure 3C). GS immunostaining was progressively lost in pericentral hepatocytes and 4 weeks after tamoxifen induction, some areas surrounding central veins were completely devoid of GS positive cells (Figure 3D). Interestingly endothelial RSPO3 seems to act at a very close range, as GS positive cells were observed side by side with negative cells. *In situ*

hybridization demonstrated the presence of both recombined and non-recombined cells in the same vein (Figure S2E), an observation that might account for the heterogenous GS staining pattern. The absence of GS positive cells was not due to apoptosis of pericentral hepatocytes, as demonstrated by histological analysis (Figure S2C and D) and the lack of activation of caspase 3 (data not shown). qPCR analysis confirmed the decreased expression of other zonated genes such as *Oat*, *Cyp7a1* and *Rdh9* (Figure 3E). The involvement of *Rspo3* in maintaining the metabolic functions of the liver was further evidenced by a decrease of the amount of direct (conjugated) bilirubin, the conjugation of which is performed in pericentral hepatocytes (Figure S2E).

In adult livers, E-cadherin expression is restricted to periportal hepatocytes. *Rspo3* deletion induced a progressive expansion of the periportal field, as evidenced by a dramatic increase in the number of hepatocyte layers displaying E-cadherin labeling. Expression of other periportal genes such as *Cyp2f2* and *Hsd17* showed an equally dramatic upregulation (Figure 3F and G). The observed phenotype mimics the changes previously observed in livers from β-catenin KO mutants thus confirming that RSPO3 is required for Wnt/β-catenin signaling during adulthood. Thus liver metabolic zonation is driven by an angiocrine factor - RSPO3 - that determines Wnt/β-catenin mediated gene expression.

Ectopic expression of *Rspo1* is sufficient to induce β-catenin signaling and aberrant metabolic zonation

We next investigated whether Rspondins might be sufficient to imprint a central fate on all hepatocytes. Both *in vivo* and *in vitro* analyses have demonstrated RSPO1 and RSPO3 to be functionally analogous in their activation of canonical β-catenin signaling. Indeed, stimulation of the AML12 hepatocyte cell line with recombinant RSPO1 and RSPO3 resulted in increased activation of the Wnt/β-catenin signaling pathway albeit with somewhat lower efficiency (Figure S3A). We therefore decided to use a *Rspo1* gain of function (GOF) allele (Rosa26 knock in) previously developed in our group (Figure S3B).

To ectopically express *Rspo1* we used an inducible *Alb-CreERT2* system that drives CRE recombinase expression specifically within hepatocytes. 8 week old *Alb-CreERT2: R26*$^{Rspo1/+}$ old animals were treated with tamoxifen and the livers analyzed 2 and 28 days after the last injection (Figure 4A and B). *Rspo1* induction was accompanied by a dramatic upregulation of *Axin2* and *LGR5*, thus validating our hypothesis that RSPO1 is able to induce Wnt/β-catenin, when ectopically expressed in hepatocytes (Figure 4C). Following *Rspo1* activation a transient change in *Wnt9b* expression was observed that disappeared at later time points

(Figure 4C). *Alb-CreERT2; R26*$^{Rspo1/+}$ double transgenic mice showed a slight increase in liver size (Figure S3C), which might be caused by cellular hypertrophy or a slight increase in proliferation. This however did not impact on liver structure (Figure S3D). Analysis of GS distribution demonstrated an expansion of the pericentral domain, which was progressively extended to zone 2 of the liver acinus, demonstrating that RSPO1 is sufficient to activate Wnt/β-catenin signaling and therefore induce a progressive expansion of the GS+ hepatocytes (Figure 4D and E). Conversely, periportal gene expression was strongly downregulated and one month after tamoxifen injection E-cadherin was absent from all hepatocytes. qPCR analysis of additional pericentral and periportal genes confirmed that zone 2 was functionally converted into zone 1 (Figure 4F and G). These observations were further confirmed by the analysis of *Alb-CreERT2: R26*$^{Rspo1/+}$ livers induced at E16,5, in which we also observed an expansion of the pericentral fate (Figure S4).

Discussion

β-catenin signaling is a central pathway in liver biology that serves equally important functions during development, tissue homeostasis and disease (Monga, 2014). Its role in metabolic zonation is well established and both deletion and stabilization (*via* removal of its negative regulator APC) have been shown to have a dramatic impact on lobular gene expression (Benhamouche et al., 2006; Burke et al., 2009). ChIP-Seq analysis has demonstrated that β-catenin interacts with TCF4 to bind pericentral target genes including GS (encoded by *Glul*), thus demonstrating its direct involvement in transcriptional regulation (Torre et al., 2011). A recent study by Yang *et al* further supported this concept by demonstrating that conditional deletion of the Wnt receptors *Lrp5/6* in hepatocytes prevented the establishment of zonated functions (Yang et al., 2014).

In the present study we have shown that RSPO3 is specifically expressed in the endothelium of the central, but not the portal vein. Restricted expression commences prior to the establishment of zonation and deletion of *Rspo3* during late stages of development interfered with the onset of zonation, thus confirming its essential role in establishing central hepatocyte identity. Furthermore, *Rspo3* deletion had no impact on *Wnt* expression levels demonstrating that the observed effects are due to Wnt/β-catenin modulation rather than loss of Wnt ligands. Surprisingly, some pericentral genes, such as *Rdh9*, displayed little change upon *Rspo3* deletion. This suggests that additional mechanisms may contribute to regulate hepatic zonation, a hypothesis recently put forward by Gebhardt R and Matz-Soja M (Gebhardt and Matz-Soja, 2014). Although dynamics of the zonated genes during the perinatal period does not fully recapitulate that of Wnt/β-catenin activity, qPCR analysis of the known β-catenin targets *Axin2*, *Oat* and *Cyp2f2* confirms the strong impact of *Rspo3* deletion or activation of *Rspo1* on Wnt/β-catenin driven transcriptional regulation.

The fact that loss of Rspo/Wnt/β-catenin signaling prevents the activation of the pericentral differentiation suggests that portal identity is the hepatocyte default state. Indeed, immediately after birth hepatocytes are periportal like and although Wnt/β-catenin signaling is fully established by P10, the expression of periportal markers such as E-cadherin (also considered as Wnt/β-catenin buffering system) is still retained in most hepatocytes indicating that pericentral differentiation is a form of specialization that allows the organism to cope with changes in metabolic requirements.

By adulthood metabolic zonation is firmly established, with precise gene signatures distinguishing zone 1 and zone 3 hepatocytes (Braeuning et al., 2006). The expression of *GS* and *Cyp7a1* (and *Rdh9*) closely follows that of the classical Wnt/β-catenin targets *Axin2*

and *Lgr5* thus further supporting the concept that Wnt/β-catenin is the master regulator of liver zonation. While this manuscript was under revision, a paper by Wang et al. reported that *Wls,* a gene essential for the sorting and secretion of WNT proteins, is required within the endothelium to drive β-catenin induced cell renewal in central hepatocytes and establish zonation (Wang et al., 2015). The observed phenotype in our mutants is similar, but stronger than that observed by endothelial deletion *Wls* demonstrating that *Rspo3* plays a crucial role in the regulation of Wnt/β-catenin signaling. The authors further identified both *Wnt2* and *Wnt9b* to be specifically expressed in endothelial cells of the central vein (Wang et al., 2015), a finding that nicely confirms our own analysis. While a specific deletion of *Wnt2* and *Wnt9b* was not performed in this study, it is likely that these two genes act together with *Rspo3* to establish and maintain liver zonation.

Ectopic expression of *Rspo1* throughout the liver acinus resulted in the transformation of zone 2 hepatocytes to a pericentral (zone 3) identity, as evidenced by the expansion of the number of GS+ hepatocyte layers. Surprisingly, hepatocytes close to the periportal system failed to express GS. This is in contrast to mice carrying hepatocyte-specific deletion of APC, a negative regulator of β-catenin signaling (Benhamouche et al., 2006). APC levels are higher in periportal hepatocytes (Benhamouche et al., 2006) and it is conceivable that β-catenin activation by the *Rspo1 GOF* allele was insufficient to completely overcome the periportal APC activity. Of note, RSPO1 is less efficient in supporting canonical β-catenin signaling when compared to RSPO3 in cell culture systems (Kim et al., 2008). Similarly in AML12 cells, recombinant RSPO1 required ten times higher concentrations than RSPO3 to induce similar levels of *Axin2*.

An alternative explanation for the conversion of zone 2 hepatocytes in Rspo1 overexpressing mice could be the expansion of the recently identified Axin2+ progenitors (Wang et al., 2015) that in combination with a permissive signaling environment (high Rspo1 levels) would keep their identity while being displaced along the central-portal axis. Although plausible, this hypothesis has several pitfalls: 1) the expansion of the GS+ domain occurs in a reasonably short time frame when compared to the time required for Axin2+ progenitors to replace the liver parenchyma (Wang et al., 2015); 2) while periportal hepatocytes fail to activate the Wnt/β-catenin target GS, they do respond to *Rspo1* upregulation, as evidenced by the loss of E-cadherin expression; 3) long term follow up of the GOF animals (9 months) fail to demonstrate an expansion of the GS+ hepatocytes towards the portal vein. These observations suggest that *Rspo1* is inducing Wnt/β-catenin signaling, probably by amplifying endogenous low level Wnt signals that are absent from periportal hepatocytes. Alternatively,

a counteracting signal, potentially released from the periportal triad, could interfere with full-blown activation of β-catenin signaling in our gain of function model.

Rspondins are generally considered as facilitators of Wnt/β-catenin signaling that exert their function by binding to LGR receptors, most notably LGR5. LGR5 expression appears to be very low in the liver and we were unable to detect expression by ISH or antibody staining. Similarly, lineage-tracing experiments using an LGR5-CreERT2 line did not mark central hepatocytes (Huch et al., 2013). While expression appears to be low, differential microarray analysis of central versus portal hepatocytes identified *LGR5* to be enriched in central hepatocytes, which is consistent with an RSPO3-LGR5-β-catenin cascade in this compartment. Wang *et al* recently performed a lineage tracing analysis of Axin2+ hepatocytes and demonstrated that under physiological conditions, pericentral hepatocytes function as progenitors that replace the parenchyma after one year. They further reported the existence of a progenitor niche provided by endothelial *Wnt2* and *Wnt9b* (Wang et al., 2015). Interestingly, loss of Wnt ligands resulted in decreased, but not abolished proliferation of GS+ hepatocytes. However, the presence of GS in these pericentral hepatocytes suggests that residual Wnt/β-catenin signaling persisted in their model, which might account for the small differences observed. *Rspo3* KO animals completely lack GS expression and it would be interesting to test whether progenitor expansion would be more strongly affected in these mutants. Unfortunately, ubiquitous deletion of *Rspo3* causes mortality 6 weeks following induction thus preventing long-term analysis.

In conclusion our data imply that Rspondin activity is required for Wnt induced signaling, and may therefore be considered as a gatekeeper for the activation of the β-catenin pathway in liver hepatocytes. The specific expression of *Rspo3*, *Wnt2* and *Wnt9b* in central veins is intriguing, as they allow distinction of the endothelial compartments of the liver on the molecular level. Although several hypotheses have been put forward to explain the mechanisms responsible for the onset and maintenance of metabolic zonation (Gebhardt and Matz-Soja, 2014; Torre et al., 2010), the signal triggering Wnt/β-catenin activation has remained elusive. Potential signals may include gradients of oxygen, nutrients, metabolites, hormones and cytokines along the lobules (Gebhardt and Matz-Soja, 2014). Whatever the mechanism, it seems likely that it will directly influence and induce the expression of *Rspo3* in the endothelial compartment of central veins. The identification of RSPO3 as a key regulator of central identity may allow the identification of such signaling pathways, by monitoring the expression of RSPO3 under various conditions.

EXPERIMENTAL PROCEDURES

Mice

All animal work was conducted according to national and international guidelines. The *cCAG CreERT2*; and *Alb CreERT2* mouse lines were previously described (Hayashi and McMahon, 2002; Schuler et al., 2004). The *Rspo3* conditional allele has been generated at the Riken core facility and will be described elsewhere (details on request). *Rspo3* deletion was achieved by intraperitoneal injection (i.p.) of 5 mg of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich), per 25 g of body weight. Cre activation in the perinatal period was obtained by a single tamoxifen administration into pregnant females carrying E16.5 embryos. Cre activation in adult mice was obtained by administrating tamoxifen for every other day for one week starting at 8 weeks of age.

Immunofluorescence and Histological Analysis

For immunofluorescence experiments, tissues were fixed overnight in ANTIGENFIX (DiaPath), progressively dehydrated, and paraffin-embedded. 5 µm thick sections were rehydrated, boiled in a pressure cooker for 2 min with Antigen Unmasking Solution (Vector Laboratories), and blocked in a PBS solution containing 10% normal donkey serum (Jackson Immunoresearch) and 1% BSA and 0,1% TritonX100. All primary antibodies were applied overnight at 4^0C at the concentration listed in the Supplemental Experimental Procedures. Secondary antibodies were diluted 1:400 and applied at room temperature for 1 hr.

Glutamine Synthetase immunofluorescence after RNA in *situ* hybridization was performed without antigen retrieval. For histological analysis, tissues were fixed overnight in ANTIGENFIX (DiaPath) progressively dehydrated and embedded in paraffin-embedding media. 5 µm thick sections were then stained with Hematoxylin and Eosin (HE).

RNA In Situ Hybridization

Tissues were fixed overnight in ANTIGENFIX (DiaPath), progressively dehydrated, and paraffin-embedded. Then 7 µm thick sections were hybridized with *Rspo3* probe (details on request) according to previously described protocols (Comai et al., 2010).

Cell culture

The AML12 cell line was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40ng/ml dexamethasone and 10% fetal bovine serum, in a humidified atmosphere (5% CO2) at 37°C. All culture reagents were purchased from Invitrogen (Carlsbad, CA). All stimulations were performed according to the following procedure: cells were propagated in standard

culture medium, serum-starved for 12 h, after which recombinant mouse RSPO1 or RSPO3 proteins (R&D) were added in fresh serum-free medium. Cells were stimulated for 24h and RNA was extracted.

Liver cell isolation and flow cytometry

Livers were collected from 8 week old animals and non parenchymal cells were isolated following the protocol described by Rountree *et al* (Rountree et al., 2011). Briefly, the tissue was digested in PBS containing 5mg collagenase type I (Worthington), 5mg pronase (Roche) and 1mg DNase (Roche) for 30 min at 37C. The liver was dissociated and passed through a 70 µm filter. Hepatocytes were separated from non parenchymal cells by serial low speed centrifugations (50g for 1 min). The supernatant was then stained with CD31-647 (eBioscience). Cells were analysed with a FACS ARIA II (BD).

RT-qPCR

RNA was extracted from AML12 cells, P10 or adult livers using TRIzol reagent (Invitrogen), following the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) in combination with polydT primers. The cDNA obtained was then used as a template for quantitative PCR carried out using the Syber Green Master Kit (Roche) and a Light Cycler 480 (Roche). Expression levels were normalized for GAPDH. Primers (see primers list in the Supplemental Experimental Procedures) were designed on the Roche Universal Probe Library website.

Statistical Analysis

Statistical analysis was performed according to the Student's t test. Error estimates values are expressed as SD.

Acknowledgements

We would like to thank the staff of the animal facility for their outstanding work and dedication and Agnes Loubat for expert FACS analysis; we are grateful to Christine Perret for initial input. We are indebted to Fariba Ranc for the generation of the *Rspo1* gain of function mice and Fabio Avoglio for experimental assistance and scientific discussion. This work was supported by grants from ARC (SL22020605297), ANR (ANR-ADSTEM & ANR-11-LABX-0028-01) and the FDF (Fondation de France) to A.S.; A.S.R. was supported by a fellowship from ARC (Association pour la Recherche sur le Cancer). T.K. was supported by a fellowship from the Welcome Trust.

Author contributions

A.S.R. and A.S. designed the project. A.S.R. carried out all experiments, if not otherwise stated. V.V. generated the *Rspo1* gain of function allele and performed RNA *in situ* hybridization. M.M. provided expertise in imaging. A.C. provided support for managing the mouse colony. T.K. analyzed histological analysis of liver sections. H.O. provided the *Rspo3* floxed allele. A.S.R. and A.S. wrote the manuscript, and all authors provided editorial input.

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Figure legends

Figure 1: *Rspo3* is an endothelial marker displaying liver patterning.

(A) *Rspo3 in situ* hybridization and immuno-labeling of Keratin 18 (green) in E17.5 livers. *Rspo3*-positive endothelial cells were restricted to the central vein, as evidenced by the lack of a differentiating ductal plate labeled by Keratin 18 expression (middle panel). **(B)** *Rspo3* positive cells (blue) are detected in the central vein endothelium in P10 and 8-week old animals. Few *Rspo3*+ cells can be found in the liver sinusoids in the vicinity of central veins. The *Rspo3* expression domain overlaps with that of zone 3 hepatocytes as evidenced by Glutamine Synthetase labeling (green). See also Supplementary Figure 1. **(C)** qPCR analysis demonstrating *Rspo3*, *Wnt2* and *Wnt9b* enrichment in CD31+ sorted endothelial cells. * $p< 0.05$; **$0.05< p< 0.001$, *** $p< 0.001$ using student T test. All scale bars represent 50µm; error bars represent SD.

Figure 2: *Rspo3* is required for the onset of metabolic zonation.

(A) Schematic representation of the genetic approach used to delete *Rspo3*. Red

arrowheads indicate LoxP sites. **(B)** Tamoxifen induction protocol used to delete *Rspo3* in the perinatal period. **(C)** qPCR analysis demonstrating efficient *Rspo3* deletion and an 80-90% downregulation of the Wnt/β-catenin target genes *Axin2* and *LGR5*. **(D)** GS expression (red) is lost in pericentral hepatocytes following *Rspo3* deletion. The endothelial Wnt ligands *Wnt2* and *Wnt9b* remained unchanged. Portal tracts can be identified by bile ducts labeled by Krt18 (green). **(E)** qPCR analysis of additional Wnt/β-catenin target/zonated genes demonstrated downregulation of pericentral restricted genes. **(F)** E-cadherin immunostaining (green) demonstrating a shift in its expression gradient. **(G)** Wnt/β-catenin negative targets become strongly upregulated by *Rspo3* deletion. **cv**, central vein; **pv**, portal vein. * $p< 0.05$; **$0.05< p< 0.001$, *** $p< 0.001$ using student T test. All scale bars represent 100μm; error bars represent SD. See also Supplementary Figure S2

Figure 3: *Rspo3* is required for the maintenance of metabolic zonation.
(A) Schematic representation of the genetic approach used to delete *Rspo*3 in adulthood. Red arrowheads indicate LoxP sites. **(B)** Tamoxifen induction protocol used to delete *Rspo3* in 8-week old animals. KO livers were analyzed 2 days and 4 weeks after the final injection. (**C)** qPCR analysis demonstrating partial *Rspo3* deletion, absence of changes in *Wnt2* and *Wnt9b* expression and a downregulation of the Wnt/β-catenin target genes *Axin2* and *LGR5*. **(D)** GS is expressed in a rim of hepatocytes surrounding the central vein (green), which is lost in pericentral hepatocytes following *Rspo3* deletion. Portal tracts can be identified by bile ducts labeled with Krt18 (red). **(E)** qPCR analysis of additional Wnt/β-catenin target/zonated genes demonstrated downregulation of pericentral-specific genes. **(F)** E-cadherin immunostaining (green) demonstrating a shift in its expression gradient. **(G)** Wnt/β-catenin negative targets become strongly upregulated upon *Rspo3* deletion. **cv**, central vein. * $p< 0.05$; **$0.05< p< 0.001$, *** $p< 0.001$ using student T test. All scale bars represent 100μm; error bars represent SD. See also Supplementary Figure S2

Figure 4: Ectopic *Rspo1* expression induces Wnt/β-catenin signaling and disrupts liver zonation
(A) Schematic representation of the genetic approach used to ectopically express *Rspo1* in adulthood. Red arrowheads indicate LoxP sites. **(B)** Tamoxifen induction protocol used to induce *Rspo1* expression in 8-week old animals; livers were analyzed 2 days and 4 weeks after the final injection. (**C)** qPCR analysis demonstrating *Rspo1* induction and upregulation of the Wnt/β-catenin target genes *Axin2* and *LGR5*. *Wnt2* and *Wnt9b* expression was not significantly affected by *Rspo1 expression*. **(D)** In non-induced animals, GS expression is restricted to the hepatocytes surrounding the central vein (green), ectopic expression of Rspo1 induces an expansion of the GS+ domain. Portal tracts can be identified by bile ducts

labeled by Krt18 (red). **(E)** qPCR analysis of additional Wnt/β-catenin target/zonated genes demonstrating upregulation of pericentral genes. **(F)** Lack of E-cadherin immunostaining (green) in periportal hepatocytes demonstrating inhibition of periportal specification by RSPO1. (G) Wnt/β-catenin negative targets are strongly inhibited by *Rspo1* upregulation. **cv**, central vein. * $p< 0.05$; **$0.05< p< 0.001$, *** $p< 0.001$ using student T test. All scale bars represent 100μm; error bars represent SD. See also Supplementary Figures S3 & S4.

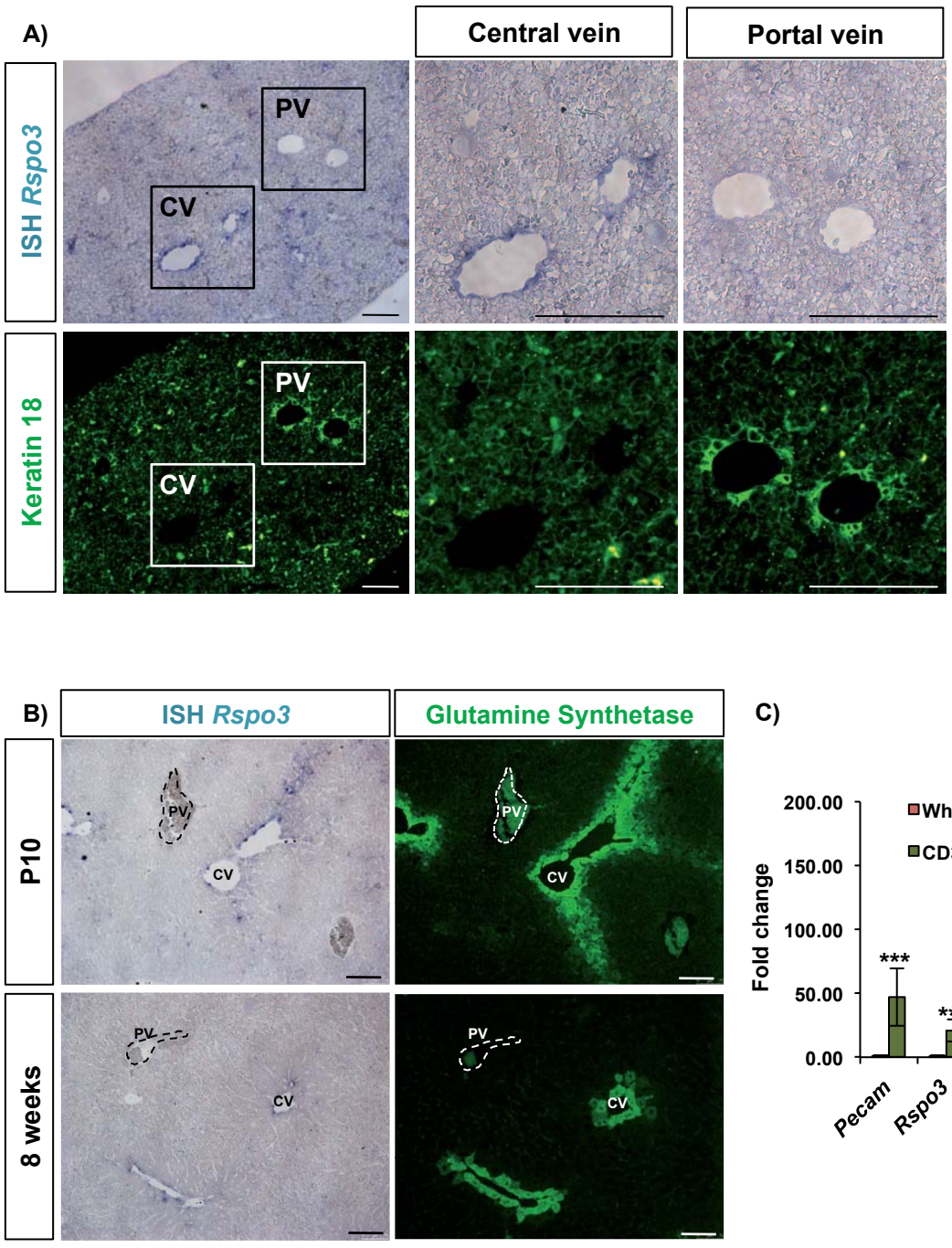

A)
Central vein
Portal vein
ISH Rspo3
Keratin 18
PV
CV
B)
ISH Rspo3
Glutamine Synthetase
P10
8 weeks
C)
Whole liver
CD31+
Fold change
200.00
150.00
100.00
50.00
0.00
***
Pecam
Rspo3
Wnt2
Wnt9b

Figure 1

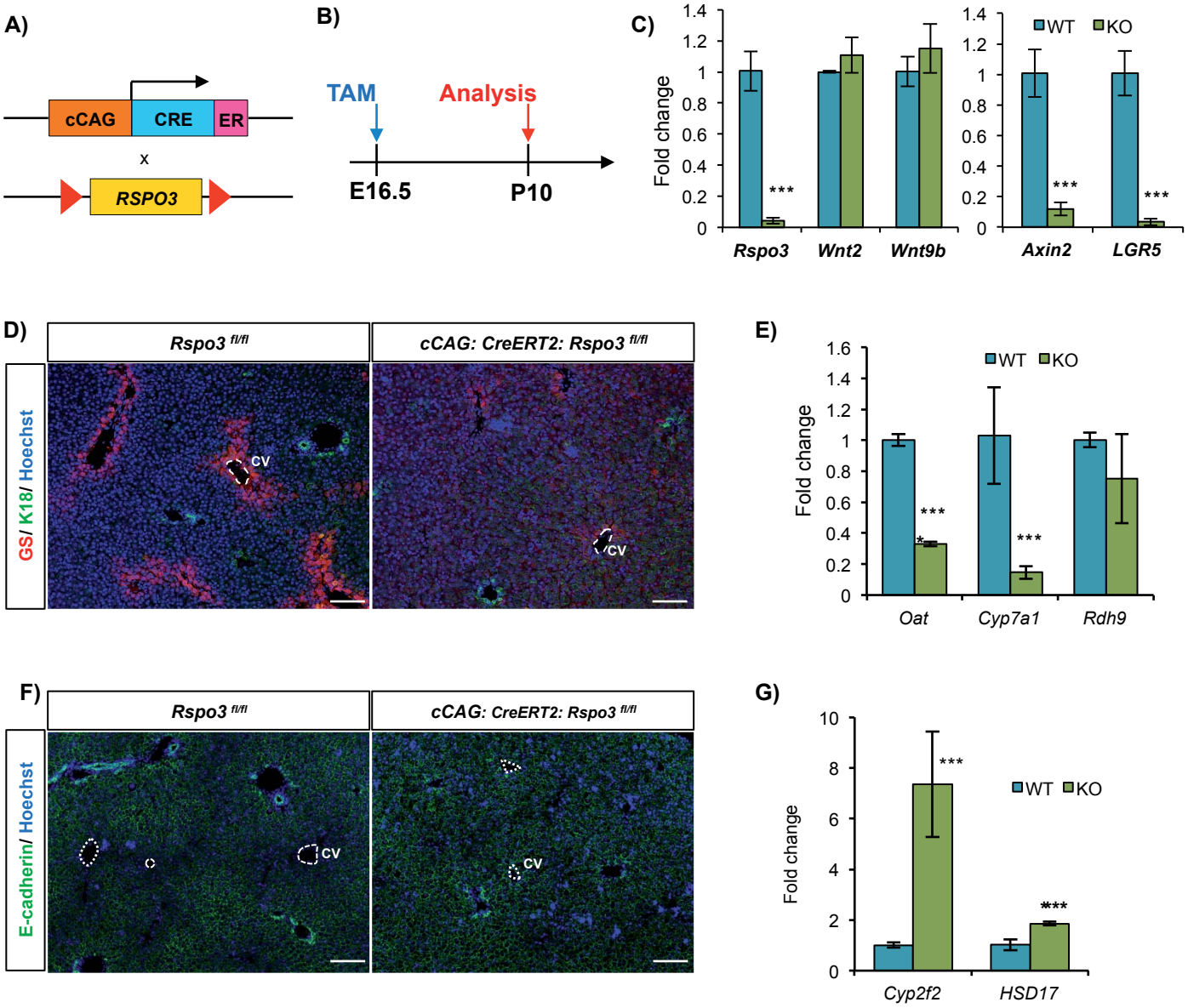

A)
cCAG
CRE
ER
x
RSPO3
B)
TAM
Analysis
E16.5
P10
C)
Fold change
WT
KO
***
Rspo3
Wnt2
Wnt9b
Axin2
LGR5
D)
Rspo3 fl/fl
cCAG: CreERT2: Rspo3 fl/fl
GS/ K18/ Hoechst
CV
E)
Fold change
WT
KO
Oat
Cyp7a1
Rdh9
F)
Rspo3 fl/fl
cCAG: CreERT2: Rspo3 fl/fl
E-cadherin/ Hoechst
CV
G)
Fold change
WT
KO
Cyp2f2
HSD17

Figure 2

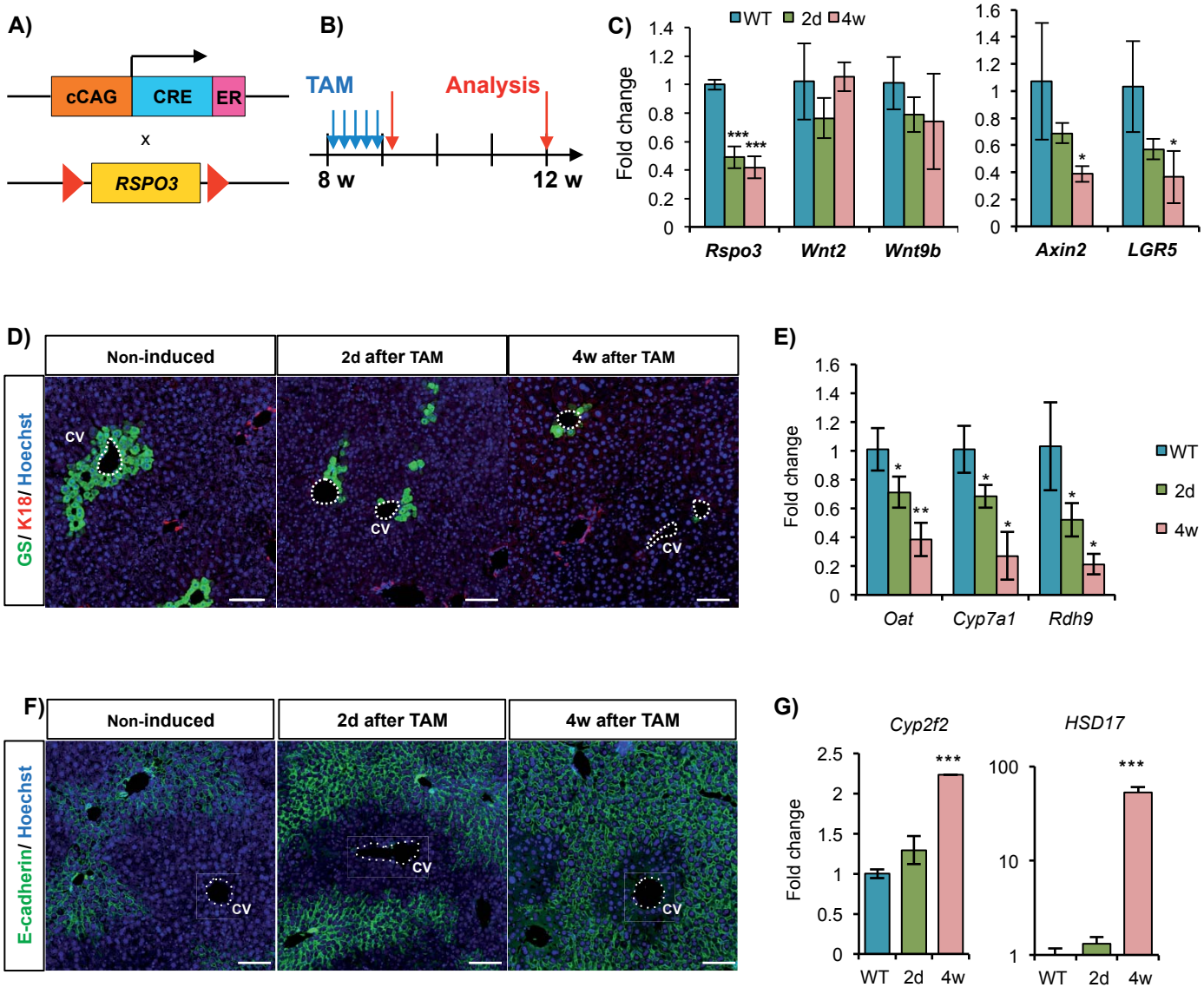

A)
cCAG
CRE
ER
x
RSPO3
B)
TAM
Analysis
8 w
12 w
C)
WT
2d
4w
Fold change
Rspo3
Wnt2
Wnt9b
Axin2
LGR5
D)
Non-induced
2d after TAM
4w after TAM
GS/ K18/ Hoechst
CV
E)
Fold change
Oat
Cyp7a1
Rdh9
WT
2d
4w
F)
Non-induced
2d after TAM
4w after TAM
E-cadherin/ Hoechst
CV
G)
Cyp2f2
HSD17
Fold change
WT
2d
4w

Figure 3

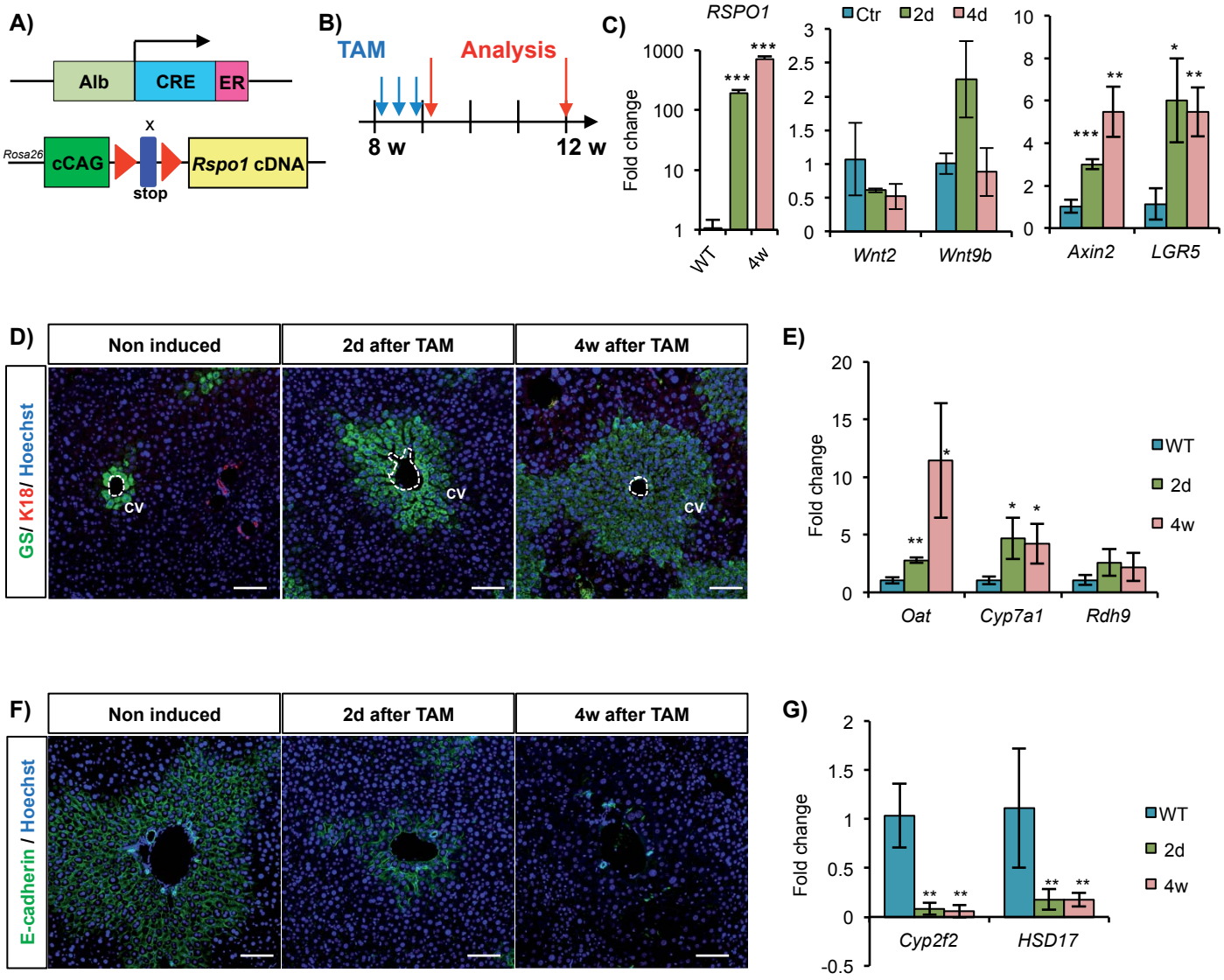
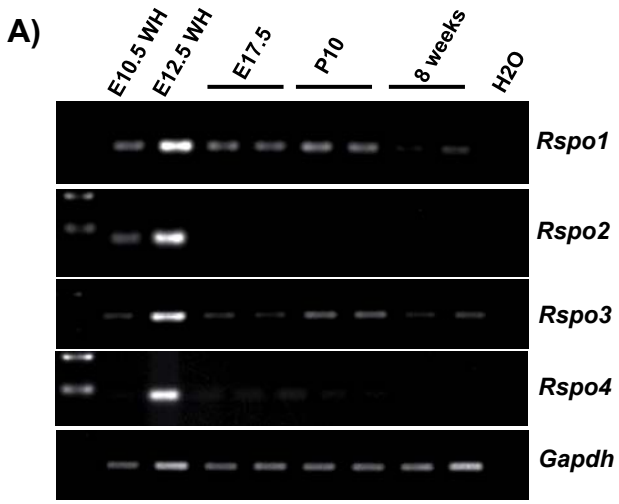
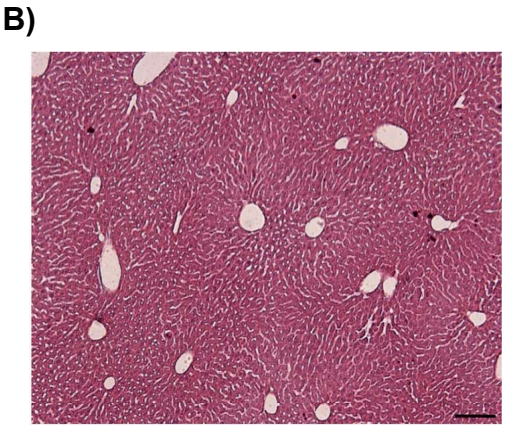

A)
Alb
CRE
ER
x
Rosa26
cCAG
stop
Rspo1 cDNA
B)
TAM
Analysis
8 w
12 w
C)
RSPO1
Fold change
WT
4w
Ctr
2d
4d
Wnt2
Wnt9b
Axin2
LGR5
D)
Non induced
2d after TAM
4w after TAM
GS/ K18/ Hoechst
cv
E)
Fold change
WT
2d
4w
Oat
Cyp7a1
Rdh9
F)
Non induced
2d after TAM
4w after TAM
E-cadherin / Hoechst
G)
Fold change
WT
2d
4w
Cyp2f2
HSD17

Figure 4


A)
E10.5 WH
E12.5 WH
E17.5
P10
8 weeks
H2O
Rspo1
Rspo2
Rspo3
Rspo4
Gapdh


B)

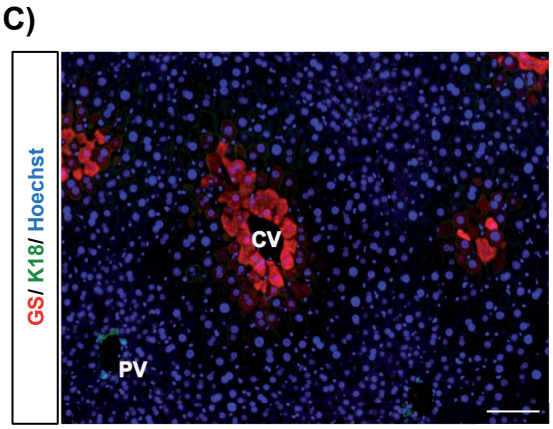

C)
GS/ K18/ Hoechst
CV
PV

Figure S1

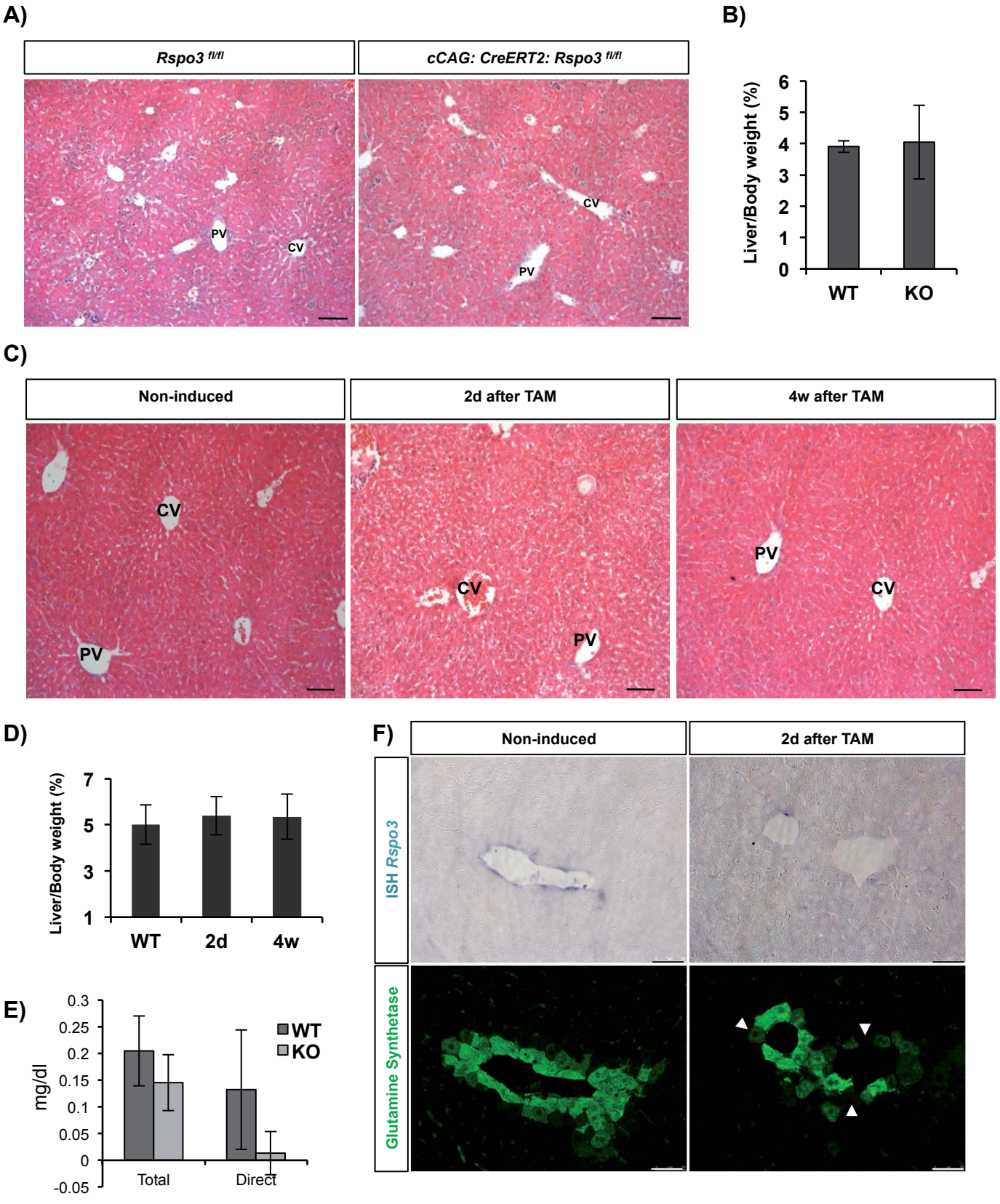

A)
Rspo3 fl/fl
cCAG: CreERT2: Rspo3 fl/fl
PV
CV
CV
PV
B)
Liver/Body weight (%)
6
5
4
3
2
1
0
WT
KO
C)
Non-induced
2d after TAM
4w after TAM
CV
PV
CV
PV
PV
CV
D)
Liver/Body weight (%)
7
5
3
1
WT
2d
4w
E)
mg/dl
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
WT
KO
Total
Direct
F)
Non-induced
2d after TAM
ISH Rspo3
Glutamine Synthetase

Figure S2

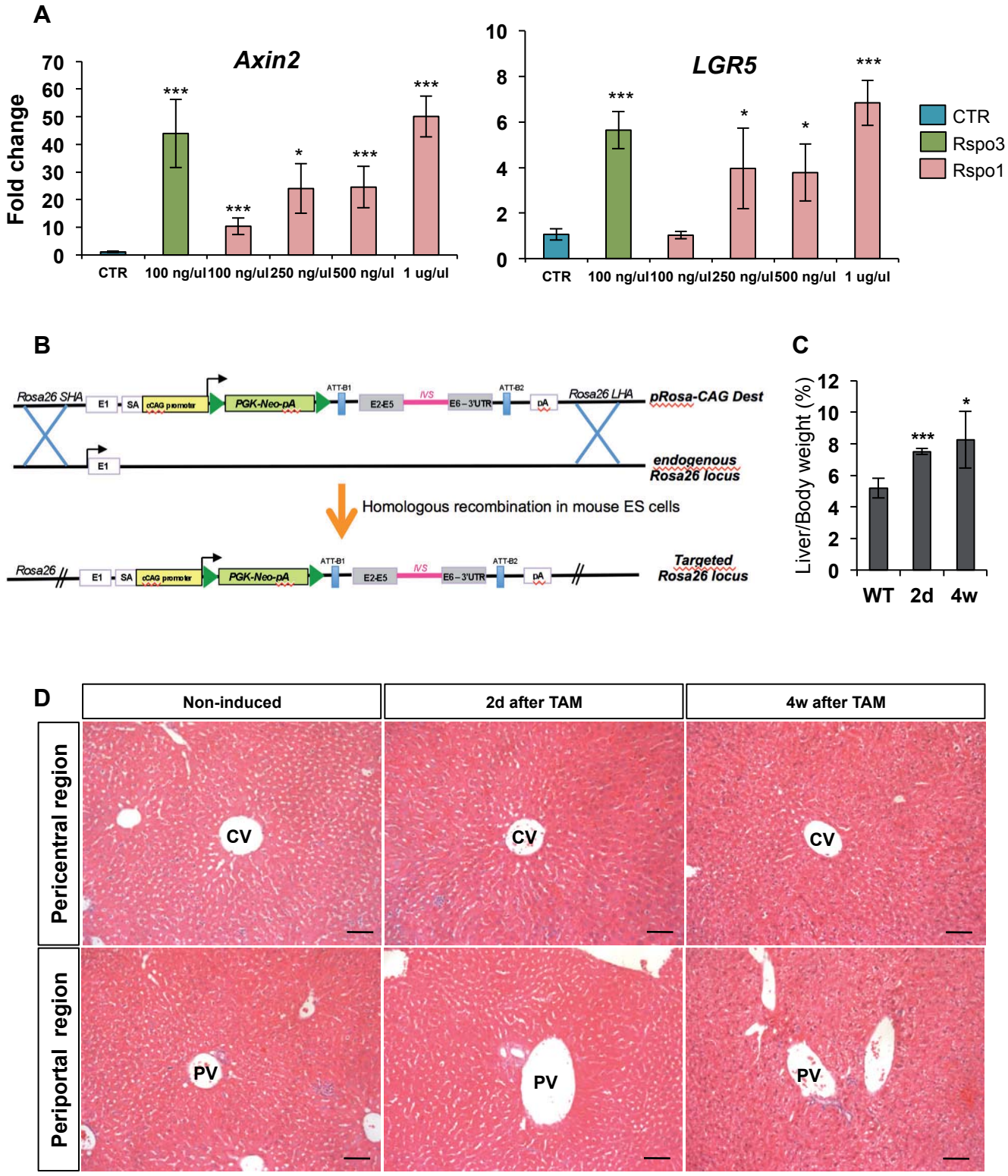

A
Axin2
LGR5
Fold change
CTR
Rspo3
Rspo1
CTR
100 ng/ul
100 ng/ul
250 ng/ul
500 ng/ul
1 ug/ul
B
Rosa26 SHA
E1
SA
cCAG promoter
PGK-Neo-pA
ATT-B1
E2-E5
IVS
E6-3'UTR
ATT-B2
pA
Rosa26 LHA
pRosa-CAG Dest
endogenous Rosa26 locus
Homologous recombination in mouse ES cells
Rosa26
Targeted Rosa26 locus
C
Liver/Body weight (%)
WT
2d
4w
D
Non-induced
2d after TAM
4w after TAM
Pericentral region
Periportal region
CV
PV

Figure S3

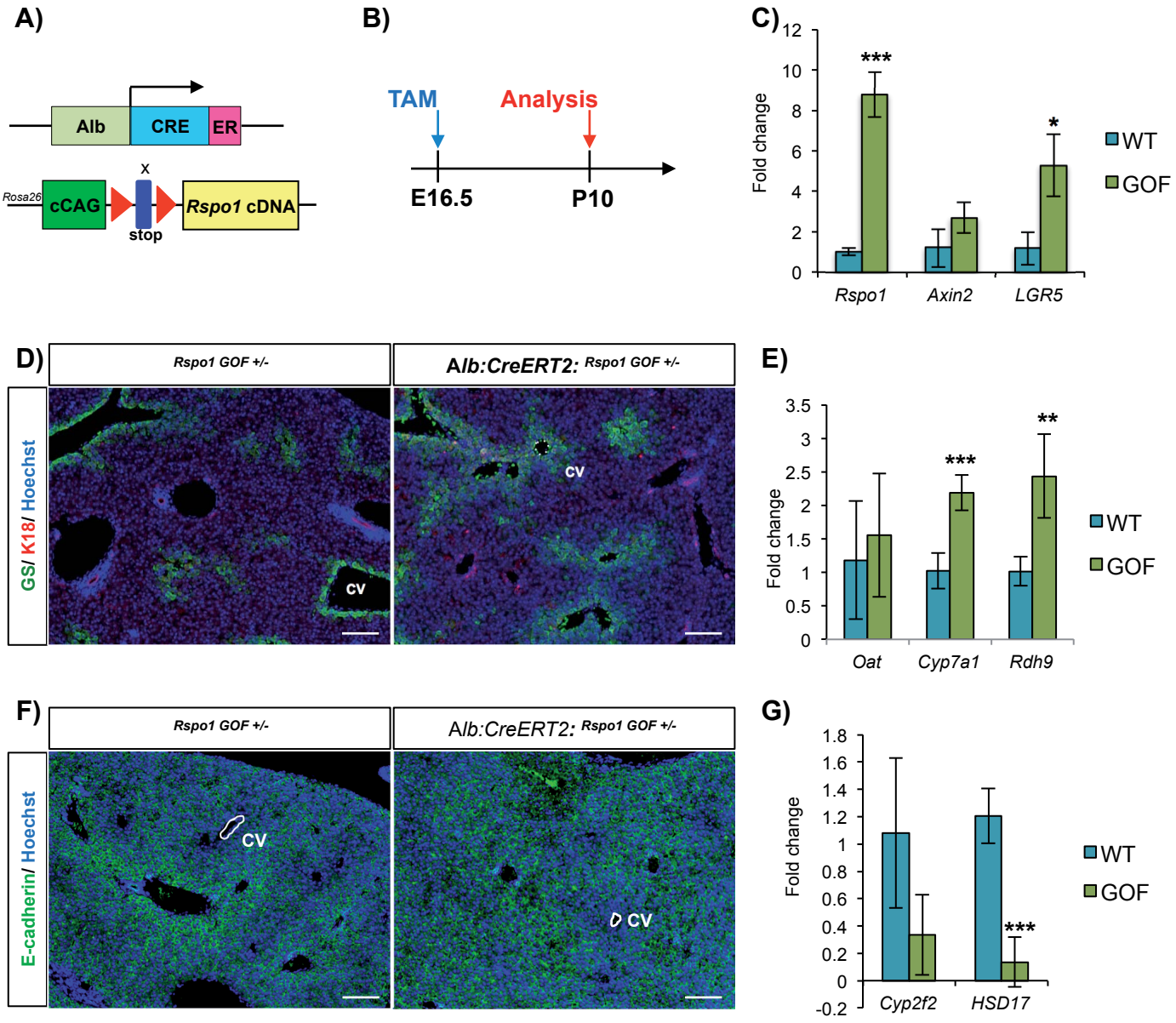

A)
Alb
CRE
ER
Rosa26
cCAG
x
stop
Rspo1 cDNA
B)
TAM
Analysis
E16.5
P10
C)
Fold change
12
10
8
6
4
2
0
***
*
Rspo1
Axin2
LGR5
WT
GOF
D)
Rspo1 GOF +/-
Alb:CreERT2: Rspo1 GOF +/-
GS/ K18/ Hoechst
cv
cv
E)
Fold change
3.5
3
2.5
2
1.5
1
0.5
0
***
**
Oat
Cyp7a1
Rdh9
WT
GOF
F)
Rspo1 GOF +/-
Alb:CreERT2: Rspo1 GOF +/-
E-cadherin/ Hoechst
cv
cv
G)
Fold change
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
***
Cyp2f2
HSD17
WT
GOF

Figure S4

Figure S1, related to Figure 1: *Rspo1, 2 and 4 are not involved in liver development and homeostasis* **(A)** Expression profile of *Rspondin* family members in E17.5, P10 and 8 week old livers demonstrating low or absent expression of *Rspo2* and *Rspo4* at all stages analysed. cDNAs from whole E10.5 and E12.5 embryos were used as positive controls. **(B)** Haematoxilin and Eosin staining of liver sections reveals normal histology in *Rspo1* KO mice. **(C)** Glutamine Synthetase labeling (red) is maintained in pericentral hepatocytes demonstrating that *Rspo1* deletion has no impact on liver zonation. Scale bar represents 50μm (B) and 100μm (C).

Figure S2, related to Figures 2 and 3: *Deletion of Rspo3 does not impair liver development and maintenance.* **(A)** Haematoxilin and Eosin staining demonstrating overall normal liver histology in *Rspo3* KO mice. **(B)** Liver/Body weight ratios at P10 remain unaltered demonstrating that *Rspo3* is not required for liver growth. **(C)** Haematoxilin and Eosin staining in 8 week old animals showing lack of major changes in liver histology in *Rspo3* KO mice. **(D)** Liver/Body weight ratios in adult mice, showing that deletion of *Rspo3* has no dramatic impact on tissue maintenance. **(E)** Billirubin quantification demonstrating defects in glucorinidation process **(F)** *Rspo3 in situ* hybridization demonstrating heterogeneous gene recombination 2 days following tamoxifen induction. GS labeling (green) showing beginning loss (white arrowheads) of pericentral identity in zone 3 hepatocytes. **cv**, central vein; **pv**, portal vein. All scale bars represent 100μm; error bars represent SD.

Figure S3, related to Figure 4: *Ectopic Rspo1 expression induces a mild hepatomegaly that is not accompanied by changes in liver structure*. **(A)** qPCR analysis of the Wnt/β-catenin target genes *Axin2* and *LGR5* demonstrating that both recombinant RSPO1 and RSPO3 are able to induce Wnt/β-catenin signaling (albeit at different efficiencies) in the AML12 hepatocyte cell line. **(B)** Schematic representation of the targeting procedure to obtain the conditional *Rspo1* gain of function allele. The cCAG promoter was inserted in front of a floxed PGK-Neo STOP cassette, which prevents expression of *Rspo1* before recombination. Cre–induced recombination results in excision of the cassette and - as a consequence - activation of *Rspo1*. *Green triangle: Lox P site, IVS: intervening sequence, Rspo1 exons are represented by grey boxes, black and pink lines indicate Rosa26 Locus and Rspo1 intron 5-6, respectively, att-B1 and attB2: Gateway cloning sites.* **(C)** Liver/Body weight ratios evidencing mild hepatomegaly 2 days following the last tamoxifen injection. No further increase in liver size was observed after an additional chase of 4 weeks. **(D)** Haematoxilin and Eosin staining demonstrating the absence of gross

histological abnormalities in *Rspo1* GOF livers. **cv**, central vein; **pv**, portal vein. * $p<$ 0.05; **0.05< $p<$ 0.001, *** $p<$ 0.001 using student T test. All scale bars represent 100μm; error bars represent SD.

Figure S4, related to Figure 4: *Ectopic Rspo1 expression disrupts the onset of metabolic zonation*. **(A)** Schematic representation of the genetic approach used to induce *Rspo1*. Red arrowheads indicate LoxP sites. **(B)** Tamoxifen induction protocol used to induce *Rspo1* in the perinatal period (2mg/25 grams of mice). (**C)** qPCR analysis demonstrating an 8 fold increase of *Rspo1* expression that is accompanied by upregulation of the Wnt/β-catenin target genes *Axin2* (4 fold) and *Lgr5 (*6 fold*)*. **(D)** GS expression (green) is expanded upon *Rspo1* induction. Portal tracts can be identified by bile ducts labeled by Krt18 (red). **(E)** qPCR analysis of additional Wnt/β-catenin target/zonated genes demonstrated upregulation of pericentrally restricted genes. **(F)** E-cadherin immunostaining (green) demonstrating a shift in its expression gradient. **(G)** Wnt/β-catenin negative targets become strongly downregulated upon *Rspo1* induction. **cv**, central vein; **pv**, portal vein. * $p<$ 0.05; **0.05< $P<$ 0.001, *** $P<$ 0.001 using student T test. All scale bars represent 100μm; error bars represent SD.

Supplementary Tables:

Antibody Table

	Host	Dilution	Producer
Glutamine Synthetase	mouse	1:1000	BD Transduction Laboratories
Keratin 18	rabbit	1:400	Abcam
E-cadherin	mouse	1:1000	BD Transduction Laboratories

Primer Table

Name	Forward	Reverse	Usage
Axin2	GCAGGAGCCTCACCCTTC	TGCCAGTTTCTTTGGCTCTT	qPCR
Cyp2f2	CCGGAACTTTGGAGGCATGAA	GGTCATCAGCAGGGTATCCAT	qPCR
Cyp7a1	CACCATTCCTGCAACCTTCT	TTGGCCAGCATCTGTAATG	qPCR
Hsd17b13	GATCCTGGAAAAAGGACCTGG	GTCTGAAGAGGGTGTCAAATCC	qPCR
LGR5	CTTCACTCGGTGCAGTGCT	CAGCCAGCTACCAAATAGGTG	qPCR
Oat	CAATTACCATCCTTTGCCTGTA	GTACTGCCTGCCTTCCACAT	qPCR
Rdh9	GAGCGTGTTGGGAACAGAG	GTTTCTTCATCCACTCGTTGG	qPCR
Rspo1	CGACATGAACAAATGCATCA	CCTCCTGACACTTGGTGCAGA	RT-PCR; qPCR
Rspo2	CCAACCAGCGAGACAAGAAC	GAGGAGGAGCTTGTTTCCTTTC	RT-PCR
Rspo3	CCAACCAGCGAGACAAGAAC	GAGGAGGAGCTTGTTTCCTTTC	RT-PCR; qPCR
Rspo4	TGTTCTTCTGCCTGGGATTT	TCTGAGTCCAGGAAGTGTCCTAT	RT-PCR
Alb:CreERT2	GGAACCCAAACTGATGACCA	TTAAACAAGCAAAACCAAAT	Genotyping
cCAG:CreERT2	CCCACCGTCAGTACGTGAGATATC	CGCGGTCTGGCAGTAAAAACTAT	Genotyping
Rspo3fl/fl	CTTCAACTTGAAGGTGCTTTACC	CCAGAATGTACAACAGATCCTCTC	Genotyping
Rspo1 GOF	CGACCTGCAGCCCAAGCTAG	TGTTCATGTCGGGGTTGCGG	Genotyping

Supplementary Methods:

Billirubin levels were quantified using the Billirubin quantification Kit (Sigma-Aldrich) according to the manufacturers instructions. Serum was collected from *Rspo3* KO animals 4 weeks following tamoxifen induction.